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Development of *Aspergillus oryzae thiA* promoter as a tool for molecular biological studies

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Abstract

In filamentous fungi, the repertoire of promoters available for exogenous gene expression is limited. Here, we report the development and application of the thiamine-regulatable *thiA* promoter (*PthiA*) in *Aspergillus oryzae* as a tool for molecular biological studies. When *PthiA* was used to express the enhanced green fluorescent protein (EGFP) reporter, the fluorescence in the mycelia was either repressed or induced in the presence or absence of thiamine in the culture media, respectively. In addition, the expression level from the *thiA* promoter can be controlled by the concentration of external thiamine. Thiamine content in the media did not affect mycelial morphology, making the *thiA* promoter more useful compared with *alcA* and *amyB* promoters that depend on carbon source for regulation. Moreover, as the *A. oryzae thiA* promoter was also regulated by thiamine in *A. nidulans*, this promoter can be further applied as an inducible promoter in other Aspergilli.

Keywords: *Aspergillus oryzae*; Thiamine; *thiA* promoter (*PthiA*); EGFP (enhanced green fluorescent protein)

1. Introduction

With the completion of the genome-sequencing project of various fungal species including Aspergilli, the cellular function of each gene product will be more extensively studied. For this purpose, the development of molecular biological techniques and tools such as promoters for exogenous gene expression for reverse-genetic approaches are, and will be, extremely important. In particular, the use of inducible promoters enables us to investigate the effects of the target gene expression directly. In addition, if the basal expression level is strictly regulated, these promoters are also suitable for expression of toxic proteins and for the generation of conditional expression mutants of genes that are essential for cell viability. In Aspergilli, the most frequently used inducible promoters are the *alcA* promoter of *A. nidulans* (reviewed in [1]) and the *amyB* promoter of *A. oryzae* [2]. These promoters, however, have common disadvantages in that their expression is regulated by the carbon source in the medium. Morphologies of filamentous fungi, such as the density of hyphae and the conidiation rate, can easily change in response to nutrients, especially to carbon and nitrogen sources (e.g., *A. nidulans* develops more aerial hyphae in ethanol medium than in glucose medium [3]). This indicates that switching the contents of the growth medium to regulate these promoters can cause potentially unpredictable and undesirable changes in cellular physiology. Thus, the use of an inducible promoter that is not dependent on

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carbon or nitrogen sources and has minimal effect on physiological conditions would be beneficial for gene expression studies. One such candidate is the promoter of *A. oryzae thiA* that encodes an enzyme involved in thiamine biosynthesis and is regulated by thiamine transcriptionally [4]. Recently, Kubodera et al. [5] reported that the expression of *thiA* was also regulated post-transcriptionally; the splicing of introns present in the 5′-untranslated region (UTR) of *thiA* and subsequent translation were blocked in the presence of thiamine. In this work, we explored the application of the *thiA* promoter as a tool for regulated gene expression in *A. oryzae*.

2. Materials and methods

2.1. A. oryzae *strains and plasmids*

Primers PthiA 5′–SmaI (5′-CCCGGGTTCGGTAAATACACTATCACA-3′, *Sma* I site is underlined) and PthiA 3′–XhoI (5′-CTCGAGGTTTCAAGTTGCAATGACTAT-3′, *Xho* I site is underlined) were used for the cloning of the 1.3 kb *thiA* promoter region (*PthiA*).

Three plasmids for the expression of *egfp* were constructed. pBNT-GFP carried 1.3 kb *PthiA* followed by 0.7 kb *egfp*, 0.3 kb *nos3′* terminator, 0.2 kb *amyB* terminator, and 5.1 kb *niaD*. This plasmid was transformed into *A. oryzae* niaD300 (*niaD*$^{-}$) using the standard *A. oryzae* transformation method [6], generating TPG1 and TPG4 strains. pUNAE that carried 0.6 kb *amyB* promoter followed by 0.7 kb *egfp*, 0.2 kb *amyB* terminator, and 5.1 kb *niaD* was constructed in this laboratory and transformed into niaD300 strain, generating NaE1 and NaE2 strains. Southern analysis of TPG1, TPG4, NaE1, and NaE2 demonstrated that a single copy of the plasmid was inserted homologously at the *niaD* locus in all four strains (data not shown). *A. oryzae* niaD300 or RIB40, the parent strain of niaD300, were used as controls. pBATG carrying 1.3 kb *PthiA* followed by 0.7 kb *egfp*, 0.3 kb *nos3′* terminator, 0.2 kb *amyB* terminator, and 2.2 kb *A. nidulans argB* was introduced to *A. nidulans* A89 (*biA1*, *argB2*) strain, yielding ATG3 and ATG4 strains.

2.2. Culture conditions

Czapek-Dox medium (CD; 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 2% carbon source, pH 5.5) was used for the microscopic observations. As a carbon source, glucose was used, unless indicated otherwise. M medium (0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 2% glucose, pH 5.5) was used for the measurement of EGFP fluorescence and RT-PCR analysis. Minimal medium for *A. nidulans* (MM) [7] containing 0.02 μg/ml d-biotin (Sigma Chemical Co., St. Louis, MO) was used for transformation and cultivation of *A. nidulans*. Thiamine hydrochloride (Sigma) was added to the media at the indicated concentrations when necessary.

2.3. Microscopic observations

Approximately 10^3 conidia, collected from a mycelium that was grown on CD agar plate containing 10 μM thiamine, were inoculated in 100 μl CD liquid medium containing 0, 0.3 or 10 μM thiamine on cover slips. Cells were grown at 30 °C for 20 h, and microscopic observation of the cover slip cultures was performed using Olympus System Microscopic Model BX52 (Olympus, Tokyo, Japan). Microscopic observation of *A. nidulans* ATG strains was performed similarly, with the exception of the cultivation medium, which was MM containing 0.02 μg/ml d-biotin.

2.4. Measurement of EGFP fluorescence of mycelia with microplate reader

Approximately 10^6 conidia of either TPG1, TPG4 or RIB40 were inoculated in 200 μl M medium containing 0, 1.0, 10, 100 nM or 1.0, 10, 100 μM thiamine in each well of 96-well microplate. The microplate was incubated at 30 °C with shaking, and fluorescence was measured every 2 h for 20 h using a spectrofluorometer (FP-6500, JASCO Co., Tokyo, Japan). The excitation and emission wavelengths were set at 488 and 510 nm, respectively.

2.5. RT-PCR analysis

Total RNA of TPG1 was extracted using Isogen (Nippon Gene, Toyama, Japan) from cultures grown for 22 h in M medium, M medium containing 10 nM or 10 μM thiamine. Total RNA of NaE1 was extracted from cultures grown for 22 h in CD medium supplemented with 1% polypeptone and 2% glycerol, glucose or dextrin as the carbon source. mRNA was purified from total RNA using Oligotex™-dT30 <super> mRNA Purification Kit (TaKaRa Shuzo, Kyoto, Japan) and subjected to reverse transcription reaction by Expand Reverse Transcriptase (Roche Diagnostics GmbH, Penzberg, Germany). Quantitative PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche) with the Light Cycler Quick System 330 (Roche). The PCR mix was subjected to the following reaction: polymerase activation at 95 °C for 60 s, and 45 cycles of PCR with denaturation at 95 °C for 15 s, annealing at 52 °C for 5 s, extension at 72 °C for 50 s, and an optical read step at 84 °C, in which only specific PCR products were detected, for 1 s. The following

7 primers were used for RT-PCR analysis (Fig. 3(A)): PthiA-N5′ (5′-AACCAACCGACAATTCATTGC-3′) was constructed based on the sequence in 5′-UTR in *PthiA* that was generated by splicing and used to amplify the spliced form of *egfp* cDNA (Fig. 3(A), a). PthiA-S5′ (5′-AAATACACTCCTCGATTAGCC-3′) was designed based on the sequence in the second intron of 5′-UTR in *PthiA* and used to amplify non-spliced form of *egfp* cDNA (Fig. 3(A), b). The primer egfp-M3′ (5′-TGTAGTTGTACTCCAGCTTGT-3′) was used to pair with the above 2 primers to amplify the *egfp* cDNA (Fig. 3(A), c). The full-length of *egfp* cDNA was amplified with EGFP 5′-EcoRI (5′-ATCGAATTCGATCCCATGGTGAG-3′) and EGFP 3′-EcoRI (5′-TCGAATTCGTACAGCTCGTCCAT-3′) primers (the *Eco*RI sites are underlined) (Fig. 3(A), d and e, respectively). γ-actin was amplified with actin-5′ (5′-GTTGCTGCTCTCGTCATTGAC-3′) and actin-3′ (5′-GTAATCGGTCAAATCACGGCC-3′) primers and used as an internal control for normalization of RT-PCR data.

3. Results

3.1. TPG strains display thiamine-dependent egfp expression

Although transcriptional *cis*-elements often exist within 1 kb upstream from the initiation codon in filamentous fungi [8], *thiA* gene has been reported to have a long 5′-UTR (ca. 500 nucleotides in length) [5]. We therefore cloned a relatively long (1.3 kb) putative upstream regulatory region and used it as the *thiA* promoter (*PthiA*) in this study. To check if this sequence is sufficient for thiamine-regulated, conditional expression of the downstream gene, we constructed *A. oryzae* strains, TPG1 and TPG4, which harbor the plasmid carrying *PthiA* followed by the *egfp* reporter gene. TPG strains displayed no distinguishable differences in morphology compared with the control strain, niaD300, neither in the presence nor absence of thiamine, suggesting that expression of *egfp* did not impair the integrity of the mycelia (Fig. 1(a)).

To study the stringency of *PthiA* regulation by thiamine, we next observed the EGFP fluorescence in hyphae of TPG strains grown in CD with or without thiamine. As shown in Fig. 1(b), EGFP fluorescence was seen in the cytoplasm in the absence of thiamine. In contrast, in the presence of more than 0.3 μM thiamine, fluorescence in TPG strains remained only at background level, suggesting an almost complete repression of *PthiA* by thiamine. Northern blot analysis in TPG strains for *egfp* expression also demonstrated similar thiamine-dependent regulation of *PthiA* (data not shown), consistent with the results reported by Kubodera et al. [4,5].

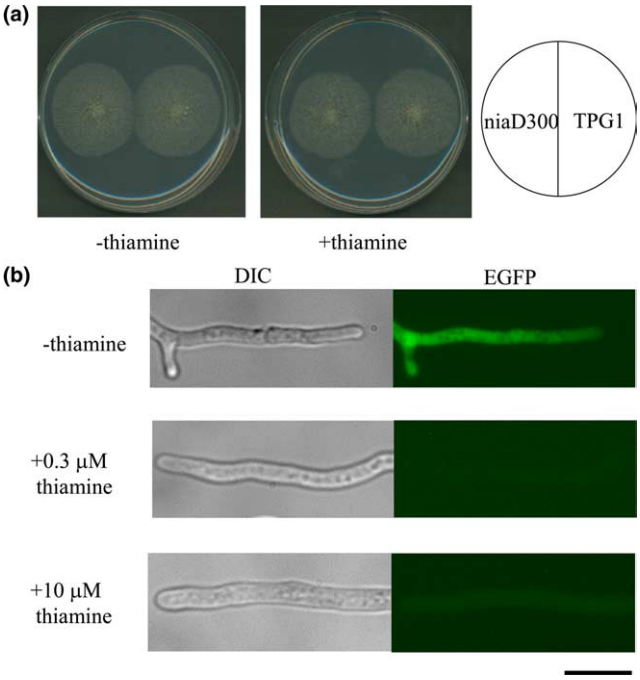

Fig. 1. Expression of *egfp* reporter gene under the control of *PthiA*. (a) To check the phenotypes of TPG, the strain expressing *egfp* under the control of the cloned *PthiA*, niaD300 (control) and TPG1 were inoculated on M medium plate and grown at 30 °C for 5 days. Left panel: CD medium; Right panel: CD medium containing 10 μM thiamine. (b) Conidia of TPG1 were inoculated in CD liquid medium containing the indicated concentrations of thiamine and incubated for 20 h. Left panels: DIC images: right panels: EGFP fluorescence. Scale bar, 10 μm.

Furthermore, the addition of thiamine into the medium did not cause changes in growth rate, mycelial or hyphal morphologies both in the control and TPG strains (Fig. 1 and data not shown). From these results, we concluded that the presence of thiamine does not significantly affect cellular activities except in the thiamine biosynthesis. Thus, *PthiA* is more favorable for exogenous gene expression than *alcA* and *amyB* promoters in that the gene regulation can be achieved without changing the fungal morphology.

3.2. Extracellular thiamine concentration affected EGFP fluorescence intensity in TPG strains

Since expression from exogenous promoters sometimes causes overexpression of the gene of interest and subsequently displays artifactual results, promoters whose expression level can be controlled are desirable. For this reason, we next measured fluorescence intensity of TPG strains in the presence of varying concentrations of thiamine using the microplate reader to investigate the correlation between extracellular thiamine concentration and the expression level of *egfp* driven by *PthiA* (Fig. 2). In the presence of more than 100 nM thiamine, relative fluorescence remained at

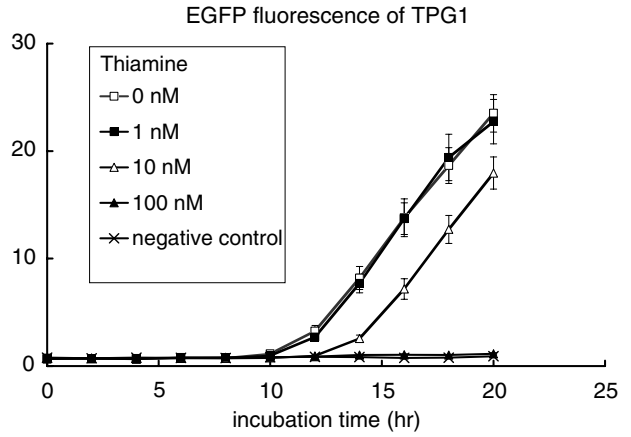

Fig. 2. Intensity of EGFP fluorescence in the mycelia grown at varying thiamine concentrations. 10^6 conidia of either TPG1 or RIB40 (negative control) were inoculated in 200 μl M medium containing 0, 1.0, 10, 100 nM or 1.0, 10, 100 μM (data not shown) thiamine in a well of a 96-well microplate. The microplate was incubated at 30 °C with constant shaking and EGFP fluorescence was measured every 2 h for 20 h. Error bars indicate standard deviations of 12 samples.

basal level in TPG1, suggesting that *egfp* expression was repressed in these conditions. In contrast, in the absence or presence of less than 1 nM thiamine, EGFP fluorescence was detected from 12 h after the inoculation and increased significantly over time. When 10 nM thiamine was present in the medium, the emergence of EGFP fluorescence was delayed by 2 h compared to the thiamine-deficient conditions, and the fluorescence remained at the intermediate level, suggesting that *PthiA* expression was mildly induced in this condition. Similar results were obtained with TPG4. These results indicate that the expression level of the gene downstream of *PthiA* can be controlled by the concentration of thiamine in the medium.

3.3. *Post-transcriptional regulation of* egfp *driven by* PthiA *was affected by thiamine concentration*

To further investigate the expression level of *egfp* driven by *PthiA*, quantitative RT-PCR analysis was performed on RNA samples collected from a 22 h culture of TPG1. Simultaneously, RT-PCR was carried out on RNA samples of NaE1, a strain that expresses *egfp* under the control of *amyB* promoter, to compare the expression level from *PthiA* to that from the *amyB* promoter. To independently detect the 5′ UTR-spliced mRNA that is subjected to subsequent translation, and the 5′-UTR non-spliced mRNA that is not translated [5], two primer pairs were constructed (Fig. 3(A) a and c, b and c, respectively).

egfp expression by the *amyB* promoter in NaE1 was the highest when dextrin was added into the medium as carbon source (Fig. 3(B), lane 12). In the presence of glucose, *egfp* expression was at intermediate level

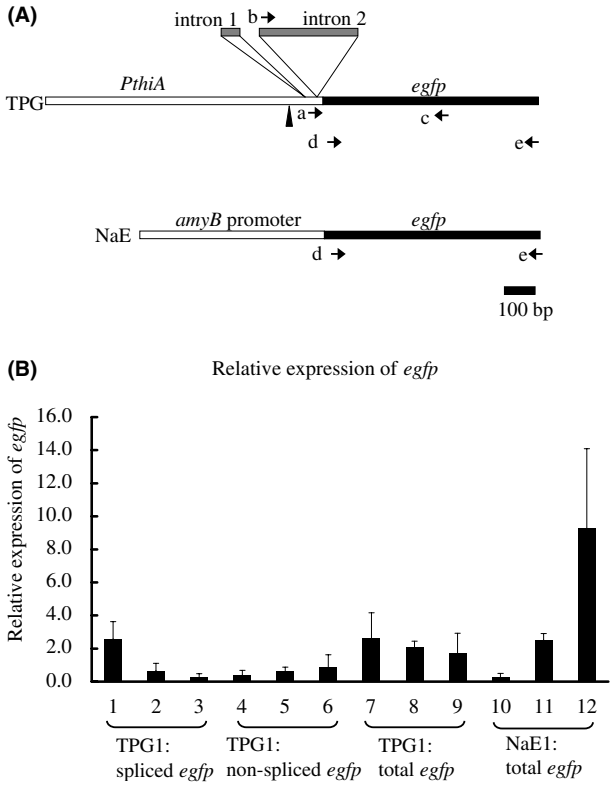

Fig. 3. RT-PCR analysis of *egfp* expression under the control of *PthiA* or *amyB* promoter. (A) Schematic illustration of the *egfp* expression system in TPG and NaE strains. Introns in 5′-UTR in *PthiA* are indicated as gray boxes. Arrows represent primers used for RT-PCR: a, PthiA-N5′; b, PthiA-S5′; c, egfp-M3′; d, EGFP 5′-EcoRI; e, EGFP 3′-EcoRI (see Section 2.5). A putative transcription start site in *PthiA* [5] is indicated by an arrowhead. (B) Relative expression of *egfp* normalized by γ-actin is shown. Lanes 1, 4, 7: M medium; lanes 2, 5, 8: M medium containing 10 nM thiamine; lanes 3, 6, 9: M medium containing 10 μM thiamine; lane 10: CD with glycerol + 1% polypeptone; lane 11: CD with glucose + 1% polypeptone; lane 12: CD with dextrin + 1% polypeptone. The graph shows the averages ± SD of two independent experiments.

(Fig. 3(B), lane 11), whereas glycerol almost completely repressed the expression (Fig. 3(B), lane 10). These results were consistent with previously reported results of the GUS reporter assay [2]. The expression of total *egfp* transcript in TPG1 in the absence of thiamine (Fig. 3(B), lane 7) was similar to that in NaE1 in the presence of glucose. In the presence of 10 μM thiamine, total *egfp* mRNA (Fig. 3(B), lane 9) was slightly lower although the difference was not significant. The amount of spliced mRNA that can be translated in thiamine-free condition was significantly higher compared to the amount in the presence of 10 μM thiamine (Fig. 3(B), lanes 1 and 3). In the presence of 10 nM thiamine (Fig. 3(B), lane 2), spliced *egfp* mRNA was found at the intermediate level when compared with other conditions, further confirming the idea that the expression of *egfp* driven by *PthiA* is moderate in this condition.

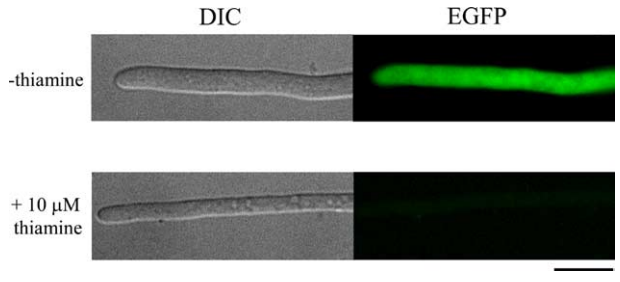

Fig. 4. Thiamine-dependent regulation of *A. oryzae PthiA* in *A. nidulans*. Conidia of ATG3 were inoculated in MM liquid medium containing 0.02 μg/ml d-biotin without or with 10 μM thiamine and incubated for 20 h at 30 °C. Left panels: DIC images: right panels: EGFP fluorescence. Scale bar, 10 μm.

3.4. Thiamine-dependent regulation of A. oryzae PthiA in A. nidulans

We next investigated if *A. oryzae PthiA* can be also used in *A. nidulans* as an inducible promoter. *A. nidulans* ATG strains that expressed *egfp* under the control of *A. oryzae PthiA* were generated and observed with fluorescence microscopy. As shown in Fig. 4, upon addition of thiamine, the EGFP fluorescence was no longer observed, results similar to those in TPG. These results indicate that *A. oryzae PthiA* was properly regulated by thiamine in *A. nidulans*, as it was in *A. oryzae*. Therefore, *A. oryzae PthiA* can be also applied as a tool for gene expression studies in *A. nidulans*.

4. Discussion

The group of filamentous fungi contain many ecologically important species in view of protein production, nutrient recycling and associations with other organisms such as human and plants [9]. In spite of their significance, molecular biological studies on filamentous fungi have been limited, partly because of the lack of adequate experimental tools and methods. Especially, to express homologous and heterologous genes by inducible promoters in *Aspergillus* species, carbon source-dependent *alcA* and *amyB* promoters have been mainly available. However, when these promoters are used for gene expression, the carbon source also affects the general morphology of mycelia while regulating the target gene expression. Here, we explored the application of the *thiA* promoter as a molecular biological tool in *A. oryzae*. The use of the *thiA* promoter is more favorable than that of *alcA* and *amyB* promoters in that the regulation of the target gene can be achieved without changing the major nutrients in medium, therefore, the effect on hyphal morphology discussed above can be ignored. This advantage is extremely important when the morphologies between the induced and repressed conditions of gene are compared directly.

The expression from the *thiA* promoter is regulated both transcriptionally and post-transcriptionally [4,5]. From our RT-PCR analysis, however, although we found the obvious post-transcriptional regulation of the transcript driven by *thiA* promoter, the transcriptional regulation was less clear. We presume that this was due to the length of the *thiA* promoter used (0.7 kb in [5] and 1.3 kb in our study). In addition, we found some nucleotide polymorphisms in *thiA* promoter between our sequence and the database sequence, and these polymorphisms could have affected the transcriptional control of the *thiA* promoter that we used. Nevertheless, as shown by microscopic observation, the microplate assay and RT-PCR analysis of spliced transcripts, the expression level of *egfp* controlled by the *thiA* promoter can be virtually completely repressed in the presence of more than 100 nM thiamine. These features make the *thiA* promoter useful for expression of toxic proteins, gene analysis and generation of conditional expression mutants. Using this system, we succeeded in obtaining a conditional expression mutant of a vacuolar syntaxin homologue in *A. oryzae* [Shoji et al., unpublished results]. Moreover, as confirmed by both the microplate assay and RT-PCR, the expression of the gene downstream of the *thiA* promoter can be maintained at the moderate level in the presence of 10 nM thiamine. Therefore, the *thiA* promoter can be further applied for the expression of genes whose overexpression causes growth defects. We have also noticed, however, a limitation in the application of *thiA* promoter: *thiA* promoter failed to be regulated properly in alkaline pH condition. Even in the presence of 10 μM thiamine, EGFP fluorescence was detected in TPG strains at pH 7 (data not shown). One possible explanation for this is that since thiamine is a weak base, loss of electronic charge at alkaline pH may perturb uptake of thiamine via a transporter. This limitation remains to be solved in future works.

Our findings indicate the great potential of the *A. oryzae thiA* promoter as a molecular biological tool in *A. oryzae*. We also found that the *thiA* promoter displayed the thiamine-dependent regulation in *A. nidulans*. Thus, it is likely that the *thiA* promoter can be also applied for gene expression studies in other Aspergilli such as *A. nidulans*, *A. fumigatus*, etc. The genome-sequencing projects of these filamentous fungi have almost been completed, which will facilitate us in investigating each organism by reverse-genetic approaches. For these studies, an efficient inducible promoter is always useful because the conditional expression techniques of various homologous and heterologous genes, such as the fusion genes with reporter genes, mutated genes and double strand RNA for RNA silencing, should be routinely performed. Molecular biological researches always require adequate experimental tools and methods. We expect that the use of the *thiA* promoter will contribute

to the progress in understanding the cellular biology of *A. oryzae* and other filamentous fungi.

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References

[1] Felenbok, B., Flipphi, M. and Nikolaev, I. (2001) Ethanol catabolism in *Aspergillus nidulans*: a model system for studying gene regulation. Prog. Nucleic Acid Res. Mol. Biol. 69, 149–204.

[2] Tada, S., Gomi, K., Kitamoto, K., Takahashi, K., Tamura, G. and Hara, S. (1991) Construction of a fusion gene comprising the Taka-amylase A promoter and the *Escherichia coli* beta-glucuronidase gene and analysis of its expression in *Aspergillus oryzae*. Mol. Gen. Genet. 229, 301–306.

[3] Som, T. and Kolaparthi, V.S. (1994) Developmental decisions in *Aspergillus nidulans* are modulated by Ras activity. Mol. Cell. Biol. 14, 5333–5348.

[4] Kubodera, T., Yamashita, N. and Nishimura, A. (2000) Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. Biosci. Biotechnol. Biochem. 64, 1416–1421.

[5] Kubodera, T., Watanabe, M., Yoshiuchi, K., Yamashita, N., Nishimura, A., Nakai, S., Gomi, K. and Hanamoto, H. (2003) Thiamine-regulated gene expression of *Aspergillus oryzae thiA* requires splicing of the intron containing a riboswitch-like domain in the 5′-UTR. FEBS Lett. 555, 516–520.

[6] Kitamoto, K. (2002) Molecular biology of the Koji molds. Adv. Appl. Microbiol. 51, 129–153.

[7] Rowlands, R.T. and Turner, G. (1973) Nuclear and extranuclear inheritance of oligomycin resistance in *Aspergillus nidulans*. Mol. Gen. Genet. 126, 201–216.

[8] Punt, P.J., Strauss, J., Smit, R., Kinghorn, J.R., van den Hondel, C.A. and Scazzocchio, C. (1995) The intergenic region between the divergently transcribed *niiA* and *niaD* genes of *Aspergillus nidulans* contains multiple *NirA* binding sites which act bidirectionally. Mol. Cell. Biol. 15, 5688–5699.

[9] Borkovich, K.A., Alex, L.A., Yarden, O., Freitag, M., Turner, G.E., Read, N.D., Seiler, S., Bell-Pedersen, D., Paietta, J., Plesofsky, N., Plamann, M., Goodrich-Tanrikulu, M., Schulte, U., Mannhaupt, G., Nargang, F.E., Radford, A., Selitrennikoff, C., Galagan, J.E., Dunlap, J.C., Loros, J.J., Catcheside, D., Inoue, H., Aramayo, R., Polymenis, M., Selker, E.U., Sachs, M.S., Marzluf, G.A., Paulsen, I., Davis, R., Ebbole, D.J., Zelter, A., Kalkman, E.R., O'Rourke, R., Bowring, F., Yeadon, J., Ishii, C., Suzuki, K., Sakai, W. and Pratt, R. (2004) Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. Microbiol. Mol. Biol. Rev. 68, 1–108.